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THE FATE OF INDIA INK INJECTED INTO THE BLOOD

I. GENERAL OBSERVATIONS

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Knowledge of the fate of foreign bodies introduced into the blood will throw light on the distribution and fate of microbes that invade the blood. In the latter part of the last century many investigators [1] made studies in this field by means of cinnabar, ultramarine blue, etc., and the results agreed in so far as the particles were arrested in the spleen, marrow, liver and lymph nodes, and mostly seen in extravascular connective tissue cells. My study concerns India ink, which consists of smaller and lighter particles than the other substances that have been used before. Some of the results were given before the Japanese Society of Internal Medicine in 1913 [2] and 1914.[3] The results obtained by Kusama [4] and also by Kiyono [5] agree in that India ink injected into the blood is deposited principally in the endothelial cells of the liver, marrow and spleen, and in the latter organ in splenocytes, while later the granules accumulate irregularly in the organs mentioned.

METHODS

A good stick of india ink is rubbed on an ink stone with a small amount of 0.8% salt solution, and this process is repeated until a considerable quantity of suspension is obtained, which is centrifugated for about 20 minutes and the upper part of the suspension filtered several times through the same paper. After sterilization by steam discontinuously 3 or 4 times, the suspension is kept closely sealed in the icebox. In my work rabbits and guinea-pigs were used and the amount injected as a rule was 0.4 c c per kilo of animal, but of course

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[1] Ponfick. Virchows Archiv, 1869, 48, p. 1. Hoffmann and Langhans, ibid., p. 303. Siebel, ibid., 1886, 104, p. 514.

[2] Nippon Naikagakkai Zassi, 1913, 1, p. 185.

[3] Ibid., 1914, 3, p. 5.

[4] Ziegler's Beiträge, 1913, 551, p. 459.

[5] Die Karminspeicherung, Jenà, 1914. Kyoto Igaku Zassi, 1917, 14, p. 198.

the actual quantity of ink injected cannot always be standardized accurately, but I took especial care to prepare enough suspension at one time for the same series of experiments. The animals were killed (blow on neck or air embolus), and all the organs and tissues were removed immediately and fixed in Zenker's fluid or formalin. The marrow was taken out carefully from the femur while certain short and flat bones were decalcified in 2% nitric acid for the examination of compact bone, joint tissue and other structures. The spinal cord and sympathetic nerves were also examined at several places. After embedding in paraffin, sections were made 5 mikrons thick; in certain cases thicker sections were used. Giemsa and Wright stains were used for the blood; hematoxylin and eosin gave the best results in the study of the ink cells and in this case the tissues were fixed in alcohol ether and destained with 0.002% HCl in 60% alcohol in order to avoid laking the blood corpuscles. The eosin staining was purposely made rather light. The only disadvantage of this method is that the granules in mast cells are not stained. It was found that Ehrlich's triple acid stain gave the best results in general.

EXTERNAL APPEARANCES

When a certain amount of ink has been injected, the conjunctiva and other visible mucous membranes as well as the skin are colored black, but after some time the color returns to normal.

NUTRITION

Reasonable amounts of ink suspension injected as described do not seem to be directly injurious to the animals. They increased in weight and size, brought forth young, their behavior in every way not differing from that of perfectly normal animals. After the injection of a large amount, e. g., over 20 c c per kilo of rabbit, no symptoms would develop, but after some days the animals would become thin and sometimes die, though that was not always the case. Rapid injection, especially of highly concentrated ink, might cause paralysis and frequently death, and in the organs of such animals the ink did not mix with the blood but formed definite emboli. The maximal dosage for rabbits was 35 to 45 c c. Guinea-pigs often died after receiving 6 to 7 c c of ink suspensions (12 to 14 c c per kilo); they evidently are less resistant to such injections than rabbits.

THE BLOOD

For a while after the injection ink granules were present in the blood, usually for about 3 hours after the usual dosage, and for about 6 to 12 hours after the injection of a larger amount. In a short time ink granules appeared in the polymorphonuclear and large mononuclear leukocytes, such granules being found only occasionally in the large lymphocytes and never in the small. In the eosinophile cells I have not found any granules in the experiment now described; the same is true of mast cells, which increase some time after the injection, but even on reinjection of ink at this time no granules were found in these cells. Nucleated red cells appeared in the blood in considerable numbers, especially after injection of large amounts of ink suspension. The table shows the percentage of ink containing cells and the number of nucleated red cells.for each 500 leukocytes in various stages after the injection. When large amounts were injected, the polymorphonuclear ink cells were most numerous 12 hours after the injection, and after the usual amount their maximum was reached in about 6 hours. In both cases the number decreased markedly between 24 and 48

TABLE 1

PERCENTAGE OF INK CELLS AFTER INJECTIONS OF INK

Rabbits	Cells	3 Hrs.	6 Hrs.	12 Hrs.	24 Hrs.	2 Days	4 Days	7 Days	14 Days	21 Days	1 Mo.	2 Mos.
I 1905 gm. 30 c c ink suspension	Polymorphonuclear ink cells	17.3	21.8	24.2	19.8	4.9	3.9	0	0	0	0	
	Mononuclear ink cells	10.0	23.0	38.0	5.0	18.8	24.2	3.5	0.7	0.4	3.1	2.0
	Large lymphocytes	0	0	0	0	1.0	1.0	0	0.6	0	0	0
	Nucleated red cells	18.0	101.0	89.0	28.0	10.0	7.0	6.0	2.0	1.0	0	
II 1775 gm. 25 c c ink supsension	Polymorphonuclear ink cells	....	29.0	35.1	19.3	3.0	1.1	0	0	0	0	0
	Mononuclear ink cells	....	7.8	36.2	64.4	25.1	13.0	4.9	2.5	0	3.2	3.6
	Large lymphocytes	....	0	0	0	0	0	0	0	0	0	0
	Nucleated red cells	....	109.0	90.0	31.0	10.0	7.0	8.0	3.0	0	1.0	
III 2055 gm. 7 c c ink suspension	Polymorphonuclear ink cells	7.8	18.5	17.0	12.0	2.4	0.5	0	0	0	0	0
	Mononuclear ink cells	21.2	7.1	0	4.9	6.4	0.7	2.9	1.9	0	2.8	1.8
	Large lymphocytes	0	0	0	0	0	0.4	0	0	0	0	0
	Nucleated red cells	21.0	61.0	45.0	12.0	4.0	3.0	1.0	1.0	3.0	0	
IV 2605 gm. 9 c c ink suspension	Polymorphonuclear ink cells	4.2	14.3	11.1	2.4	0.5	0	0	0	0	0	0
	Mononuclear ink cells	19.0	0	17.0	2.4	5.0	6.5	3.2	1.7	0	0.6	1.0
	Large lymphocytes	0	0	0.8	0	0	0	0	0	0	0	0
	Nucleated red cells	....	76.0	52.0	14.0	4.0	4.0	1.0	0	0	0	

The number of nucleated red cells indicates the number of such cells for each 500 leukocytes.

hours after the injection, and in 4 to 7 days all such ink containing cells had disappeared as a rule; in one instance they persisted for 3 weeks. The mononuclear ink cells fluctuated much more, but were present in fairly good numbers for 4 to 7 days, when a marked drop occurred, some however persisting for even over a year, and possibly during life. One rabbit showed one mononuclear ink cell of 74 counted and another one in 192, one year after the injection. In late periods the granules in mononuclear cells were always few. These results accord quite well with those obtained with cinnabar by Ponfick and by Hoffmann and Langhans. The number of granules in the polymorphonuclears usually varied from 1 to 30 or more, the cells sometimes being filled to distention. In all cases the granules were scattered through the cytoplasm, appearing only occasionally in the nucleus.

Shortly after the injection a considerable number of nucleated red cells and polychromatophile red cells appeared in the blood. Kusama made similar observations. The number of such cells was at a maximum in 6 hours, and then there was a gradual decrease, but the cells could be found 4 and 7 days after injection. With larger doses of ink, various abnormal cells made their appearance, often containing ink granules as did also the nucleated red cells. Schulze,[6] Arnold[7] and others found granules in the red corpuscles of the frog treated with methylene blue. Rost[8] found that corpuscles in frogs subjected to vital staining would contain granules more frequently when some injurious agent was injected. Recently Kiyono and Chuin[9] stated that primitive erythroblasts of higher vertebrates are capable of a slight degree of phagocytosis. Hence, it seems not unlikely that the phagocytosis of ink granules may be the result of acute regenerative processes in connection with the red cells. The relation of these red cells to so-called pseudolymphocytes and other questions are reserved for further investigation.

INTERNAL ORGANS

After death the liver, spleen and marrow were intensely black. Some lymph nodes, especially about the stomach and along the jugular vein, were also blackened, and this is true as well of the omentum and periosteum, especially after large doses and late after the usual doses of ink. Sometimes the small intestine and the lungs were stained blackish after large doses. In the case of the periosteum, it was usually in the long bones (femur, tibia, ribs) that it showed blackening, usually some weeks or months after the injection. In the flat or short bones such a change was less marked, the scapula, however, often showing some staining.

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[6] Anat. Anzeiger, 1887, 2, p. 684

[7] Virchows Arch., 1899, 157, p. 429; Arch. f. Mikr. Anatomie, 1898, 52, p. 523.

[8] Arch. f. d. gesam. Physiol., 1911, 137, p. 359.

[9] Nisshin Igaku, 1918-19, 8, p. 475.

In the liver ink granules were deposited in the endothelial cells of the blood capillaries, in the earlier stages in regular distribution giving a radial arrangement under low power from the central vein to the periphery of the acinus. Under higher power the content of different cells in granules varied, some cells being stuffed with granules and roundish from having drawn in their processes. Such cells often look as if about to fall off into the blood. Kiyono noted this also. The ink granules tended to accumulate around, or at the end of, the nucleus and in the early stages the granules were round and smooth but later they became irregular and apparently formed aggregates. Glisson's capsule and the liver cells proper did not contain any granules in the early stages after the usual dosage. About a week after the injection the amount of granules seemed to increase in some of the cells and collections of such endothelial cells began to form, the distribution of the granules being much more irregular than at first. In late stages there were large masses of cells filled with granules, such masses apparently increasing in size at the same time as granules containing cells from other parts of the liver decreased. After a year or so such masses might be detected with the naked eye when the slides were held against the light and in such cases there were only a small number of phagocytic cells in the substance of the liver elsewhere. In the course of their accumulation, the ink granules appeared to proceed from the center to the boundary of the acini, hence in the later stages the granules were found mostly at the border or in Glisson's capsule. Usually it took several days or a week before the granules appeared in Glisson's capsule. Giant cells were often found in and about accumulations of granules, but no special cell proliferation seemed to develop around the masses except that late in the process Glisson's capsule seemed to become somewhat thicker. After large doses of ink, granules would accumulate more quickly and even enter liver cells, and this seemed to be the case especially when several injections were made into the same animal. In two healthy rabbits ink granules appeared within the liver cells, in one case, the rabbit weighing 2,150 gm., after injection of 4 c c of ink suspension every day for 6 days and in the case of the second animal, which weighed 2,000 gm., after the injection of 7 c c of suspension on 4 different occasions in the same week. Kiyono reports a similar observation. Feeble rabbits, afflicted with coccidiosis, frequently presented an irregular distribution of endothelial cells and also the formation of giant cells by the coalescing of endothelial cells, resulting not only in an irregular distribution of the granules from the first, but also in the appearance of granules in the liver cells at an early period.

In the liver cells the granules were usually smaller and more uniform in size than in the endothelial cells, usually being scattered in the body of the cells. Such phagocytic liver cells were distributed irregularly.

In the spleen the endothelial cells of the capillaries and sinuses, as well as the splenocytes, took up ink granules after the usual dose. The malpighian bodies were practically free from ink in the early stages, but in feeble animals or after large doses of ink there would be some granules here also. The granules in the spleen seemed to vary in size more than those in the liver, and in the spleen also the granules subsequently migrated and eventually accumulated in larger masses so that after a year or so the masses could be seen with the naked eye. There seemed to be no special place in which such masses would form by preference. Giant cells of the foreign body type and large phagocytic cells were present in such masses, but there was no special cellular changes at their periphery. The spleen seemed to acquire more granules as time went on while in the marrow as well as in the liver the amount of ink granules seemed to decrease. In sections of the spleen of animals killed immediately after the injection of ink the capillaries could be traced easily by means of the ink granules in the endothelial cells. In the malpighian bodies a few capillaries were found in this way, but the endothelial cells here seemed to have less avidity for the ink. In the central artery there seemed to be almost no phagocytosis. The plasma cells present in small numbers in the spleen gave no evidence of phagocytosis. In special cases of animals injected with streptococci or streptococcus products, there was a marked precipitation of granules in the borders of the malpighian bodies, suggesting that phagocytic cells may develop from the reticulo-endothelial cells and passing from the center toward the periphery.

In the marrow the ink granules were deposited first in the endothelial cells of the capillaries and veins. In the red marrow, which is rich in veins, the venous network was outlined distinctly. In the early stages no granules were found in the marrow cells proper, but later the granules formed accumulations in masses, just as they did in the liver and spleen, the endothelial cells gradually becoming free. At this time the reticular cells contained granules. The osteoblasts and osteoclasts also took up granules, especially after large doses, but the myelocytes and megakaryocytes remained free.

As a rule, the bone tissue was free from granules in the earlier stages after the usual dosage but later ink granules appeared not only in the Haversian and the Volkmann canals, but also in the bone cells, although to a limited extent only, the granules being small in size and round.

At first the periosteum remained free from granules, but after a time granules were deposited to such an extent as to be detectable with the naked eye, and microscopically the granules were contained in the endothelial cells especially of the lymph vessels. After large doses the deposit occurred earlier and in greater degree.

Ink granules were also deposited in the vertebrae and in the scapulae.

Granules were found in the endothelial cells of the suprarenals, the amount depending on the amount of ink suspension injected. In from a few weeks to months the amount of granules in the suprarenals seemed to increase, the fascicular zone containing perhaps the most. After this they accumulated more particularly in the medulla, in which small collections formed. Feeble rabbits and rabbits injected with streptococci seemed to contain early more granules in the suprarenal than normal animals.

In the kidney granules were deposited to a limited extent in the cortex, especially in the glomeruli, but only after considerable doses. In late stages the granules had disappeared, except for traces in the glomeruli.

In the lungs, after moderate doses of ink suspension, granules were found in the endothelial and mononuclear cells, but no ink emboli were found except after large injections. In these organs the amount of granules found was always rather small and only a few masses. Granules were found also in the epithelial cells of the alveoli, but only occasionally. Eventually the granules of ink disappeared almost wholly from the lungs.

In the early stages and after the usual dose, the omentum and peritoneum commonly remained free from ink. In omentum majus ink granules often appeared in the middle and later stages and in feeble rabbits as well as after large quantities deposits also occurred earlier. The granules here were located in the surface cells and also in the clasmatocytes. In two rabbits with coccidiosis and ascites ink granules appeared in the abdominal cavity, the ascitic fluid becoming blackish in a short time after the injection of 25 and 30 c c of suspension; in healthy animals the granules appeared only in the endothelial cells of the capillaries even after large doses. The results in feeble animals, after intravenous injection, resembled very much the result after the intra-abdominal injection of a small amount of ink. In later stages the granules gathered in the perithelial tissue and surface cells, forming accumulations without any definite arrangement except about taches laiteuses.

Ink granules also appeared, but only to a limited extent, in the endothelial cells of the blood vessels in general, mostly in the venous capillaries. The cells of the endocardium frequently contained granules, especially in the middle periods after the injection, being situated usually about the ends of the nuclei, very small and as a rule round. In the capilaries of the myocardium granules were found, as a rule, but in the later stages they disappeared.

Lymph Nodes: Sometimes a small quantity of ink appeared in the early stage after the usual dose, and later there was often found a greater accumulation, especially in certain nodes such as the cardiac, hepatoduodenal and along the jugular. These nodes were often quite black even months after the injection. The granules were deposited in groups in the cortex as well as medulla and in such masses were found giant cells, mostly at the outskirts. At first the granules appeared in the endothelial cells of the capillaries and lymph sinuses but only when large doses were injected.

Of all the capillaries, those of the villi of the intestine seem to have more ink granules in their endothelial cells and in consequence after a large dose of ink suspension the small intestine was as a rule blackish.

In the ovary and testis a small number of ink granules were found after the usual dose. In the ovary the granules appeared in the stroma and theca folliculi; increasing somewhat with time, but I failed to find any granules in the follicular cavity. In the testis the endothelial cell as well as connective tissue cells were found to contain a moderate number of granules some time after injection, but even after large doses it was only late that a few granules were detected in the interstitial cells. Foa[10] ascribes phagocytic action to this cell while Ishibashi[11] differs on the basis of the results of injecting an emulsion of soot directly into the tissue. I shall make a special report concerning this.

In two cases I found granules in the placenta, the animals having been injected with large doses of ink suspension (1 rabbit, 1 guinea-pig). In the fetus and new-born of mothers injected with ink suspension (4 rabbits and 2 guinea-pigs) I did not find any granules in the liver or spleen.

Soon after injection clasmatocytes and connective tissue cells did not contain any granules, but later granules were often found in these cells in various parts of the body. After the injection of large doses granules appeared earlier in the cells.

In the cells proper of the brain, spinal cord, hypophysis, muscle pancreas, thyroid, salivary gland, sympathetic ganglion, etc., I failed to find any granules at any time.

When the ink is injected into the blood, granules are attracted first to the endothelial cells of the liver, the spleen, and the marrow and to the splenocytes, which cells quickly take them up. If the quantity injected is small, these cells rapidly dispose of all the ink granules. If the quantity is larger, the cells are not able to take up all the granules at once so that some remain in the blood. In the case of the largest quantity injected in these experiments, granules remained in the blood for about 12 hours. Apparently we have here an example of the phagocytic limit being reached by these cells. Other cells, such as those in the suprarenals, kidney, intestines, etc., become more phagocytic under these circumstances. When the injection of ink is repeated a wider range of cells appear to become engaged in phagocytosis. Some time after the injection accumulations of ink granules form, relieving some of the phagocytes at the same time as new phagocytic cells are formed.

In feeble rabbits replacement and accumulation of ink granules takes place more quickly than in healthy rabbits, due perhaps to degenerative and regenerative changes in phagocytic cells. It is also noticeable that the granules reached the lymph spaces more readily in feeble rabbits, as illustrated especially by the changes observed in the omentum and in ascitic fluid. The entrance of granules into liver cells may occur under the same conditions.

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[10] Quoted by Ishibashi.

[11] Mitth. ad. med. Fakultät d. kais. Univ. 3, Tokio, 1919, 22, p. 39.

Earlier investigators (Ponfick with cinnabar, Hoffmann and Langhans with cinnabar and ultramarine blue, and Siebel with indigo) observed that after intravenous injection foreign particles were deposited outside the vessels in the liver, spleen, marrow and lymph nodes. Ponfick urged that the deposition takes place directly in special cells outside the vessels, while the others asserted that there is intermediary action on the part of phagocytic leukocytes from the blood and even suggested that these leukocytes change into connective tissue cells. Kiyono ascribed an eliminating function to the liver and the other organs mentioned, foreign particles reaching the lymph spaces through the capillary walls. I agree with this view with some modification, which I shall discuss later.

The granules of ink injected into the blood seem to have no definite relation with any excretory or secretory organs and careful repeated examination of the urine and bile has not revealed any granules. After the injection of large doses, especially in feeble rabbits, granules often appeared in the liver cells proper but without any relation to the bile capillaries. It would seem as advocated by Kiyono, that ink granules can only be removed from the body by way of the leukocytes.

SUMMARY

When a suspension of India ink is injected into the blood the mucous membranes and spleen become black and remain so for variable lengths of time, depending on the amount injected.

Free granules are present in the blood for several hours when large quantities are injected, but disappear sooner after smaller quantities.

Ink granules are deposited regularly in the endothelial cells of the liver, spleen and marrow and in the splenocytes, and are eventually gathered together in accumulations of considerable extent. In the liver the granules are passed along from the centers to the borders of the acini and into Glisson's capsule. In the spleen the granules are accumulated irregularly, often at the outskirts of, or in, the malpighian bodies. In the marrow there is no special arrangement of the granules. In the peritoneum and in the osteal canals, granules appear in the later stages after the injection, some perhaps reaching the lymph spaces from the marrow. The granules found in the bone cells were very small. After large injections osteoblasts and osteoclasts may take up granules. Only a small number of granules appear in the suprarenals, kidneys and lungs. In the earlier stages there usually are few gran-

ules in the lymph node but after some time granules appear regularly in certain nodes (cardiac, hepatoduodenal, jugular) which may be quite black. Few granules are generally deposited in the blood vessels and more in the venous than in the arterial vessels. After large doses granules are found in the endocardial cells.

In the liver and spleen, as well as elsewhere, large phagocytic cells may appear, stuffed with granules, but there is no special connective tissue proliferation about accumulations of granules, in which giant cells may be found.

After large doses or repeated injections granules may appear in the liver cells proper and in the medulla of the lymph nodes, and in time aggregations of granules may form here and in the malpighian bodies of the spleen. In rabbits with coccidiosis granules may appear in the liver cells earlier than in normal animals. Only comparatively few granules are deposited in the omentum except when large quantities of ink are injected, or when the rabbit is feeble. In rabbits with ascites granules may pass into the fluid. In the ovary granules may appear in the stroma and in the theca folliculi and in the testis the interstitial cells may contain occasional granules. After large doses granules may appear in the placenta, but I did not find any ink granules in the fetus.

In late stages after the injection connective tissue and other cells in various parts of the body may contain granules, but without any regular distribution. Granules were not found in the parenchyma cells of the nervous system and various other organs with the exception of the liver and spleen.

In the blood polymorphonuclears and mononuclears take up granules; large lymphocytes do so only occasionally. The polymorphonuclear ink cells increase rapidly after the injection, reaching the maximum in 6 hours after small doses and in about 12 hours after large doses, disappearing gradually in the course of several days. The mononuclears pursue a more irregular course, such cells apparently being set free from the liver and spleen, but by the seventh day the number is greatly diminished; however, small numbers of such cells with ink granules in them may occur in the blood for as long as a year or more and perhaps during the rest of the life of the animal. After the injection a considerable number of erythroblasts appear in the blood, especially after large doses; the number continues to increase for about 6 hours when there is a rapid drop, normal conditions being reached in about a week or so. Various abnormal cells also appear in the blood, often containing ink granules, and sometimes granules are found in erythroblasts. The eosinophiles and mast cells do not take up granules.

With careful preparation of the ink suspension and care in injection, the health of the animal injected need not be disturbed, but rapid injection of large doses or highly concentrated suspension may cause death promptly, or if the animal survives it may remain feeble for several days. In such cases the animals may not live as long as those injected with smaller quantities.

Apparently ink granules are not discharged from the body by any particular organ after injection into the blood, but it is possible that they are carried to the outside by emigrating phagocytes.